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Chapter 7

PHYSIOLOGICAL AND DEVELOPMENTAL CAPACITIES OF INSECT CELL LINES

Herbert Oberlander and Stephen M. Ferkovich

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I. INTRODUCTION

Insect tissue culture has a long history beginning in 1915 when Richard Goldschmidt described his success in maintaining moth testes in organ culture.[1] Goldschmidt was at Yale at the time and was influenced by Ross Harrison who, a few years earlier was the first to employ tissue-culture methodology. Despite this early beginning, insect tissue culture attracted only a few scientists, who met with limited success with organ culture, but none at all in establishing insect cell lines. It was indeed puzzling that during the ensuing decades vertebrate cell lines became widely used, which led not a few distinguished biologists to pronounce insects incapable of generating permanent cell lines. To consider this possibility, Paul Weiss organized a conference in 1955 on the feasibility of invertebrate tissue culture and concluded that "contrary to mammalian cells, insect cells will most likely never be grown in continuous culture..."[2] Predictably, this conclusion preceded by less than a decade the report by Tom Grace of Australia in *Nature* that he had succeeded in establishing four permanent cell lines from moths.[3]

During the 1960s and 1970s there was dramatic progress in the study of physiological and developmental problems in insect organ culture, assisted in part by the availability of Grace's culture medium, among others. At the same time tremendous strides were being made in establishing vertebrate cell lines that retained capabilities reflecting their *in vivo* origin.[4] Clearly, established cell lines that retained *in vivo* properties had distinct advantages over organ culture, especially the ready availability of large numbers of cells representing a homogenous population, not to mention an end to daily dissections. Unfortunately, virtually all of the insect cell lines that were available in the 1960s were of unknown cell type, did not respond to hormones, and were used primarily for studying the propagation of insect viruses.

In later years many cell lines were tested for hormonal responsiveness, and one embryonic cell line, the Kc line from *Drosophila melanogaster,* established by Echalier and Ohanessian in Paris, was affected both morphologically and biochemically by the insect molting hormones, ecdysone and 20-hydroxyecdysone.[5,6] However, as late as 1978 the same could not be said for any of the lepidopteran cell lines. Then, in 1979 Marks and Holman observed a morphological response to ecdysteroids in a *Manduca sexta* cell line, so that hormonal effects now could be studied in both dipteran and lepidopteran cell lines.[7]

The early work on hormonal effects on insect cell lines gave hope that more complex developmental events could be investigated in cell lines established for that purpose. It is important to consider what sorts of cellular activity can be observed *in vitro* that indicate a capacity for differentiation and morphogenesis among established cell lines. Unlike organ cultures, we cannot expect an insect cell line to give rise to completely differentiated structures, tissues, or organs. Thus, we have to look for discrete steps in a developmental pathway that may only partially be implemented in cell culture. In this chapter we will discuss cell lines that demonstrate changes in cellular morphology, regulation of multicellular organization, responsiveness to ecdysteroids, and production of specific cellular products. We believe that these are legitimate examples of physiological and developmental capacities of insect cell lines, but each system must be examined critically.

In our laboratory we reasoned that if we could develop cell lines from a developmentally committed but proliferating tissue type, then we would have a better chance of observing cellular activities *in vitro* that were characteristic of *in vivo* development. To accomplish this we chose the imaginal discs. First, imaginal discs are diploid dividing cells, present in the larval stage, that are already on a developmental pathway for adult structures, but are not yet differentiated. Second, in a sense these tissues are already in culture in the larval hemolymph. They thrive there without contributing to the activities of the caterpillar in which they reside, but grow and wait for the hormonal balance to change in favor of metamorphosis. Unfortunately, imaginal discs are neither large nor numerous. However, the imaginal discs have

remarkable regulative capacities, and when wounded, proliferation is stimulated. Our laboratory took advantage of this property to establish the first cell lines from imaginal discs.[8] For the purposes of this chapter we will consider cell lines from any insect source so long as they display a recognizable developmental or physiological capacity.

II. CELLULAR ENDOCRINOLOGY

A. SYNTHESIS AND METABOLISM OF ECDYSTEROIDS

Cell lines established from imaginal discs of various Lepidoptera have shown remarkable capabilities in synthesizing ecdysteroids despite their origin from cells destined to become cuticle-secreting epithelium. For example, the imaginal disc-derived IAL-TND1 cell line, unlike other *T. ni* cell lines from ovaries or embryos, produces its own 20-hydroxyecdysone, as determined by radioimmunoassay, high performance liquid chromatography, and mass spectral analysis.[9] Moreover, Sommé-Martin et al. found that the IAL-PID2 cell line, which is also derived from imaginal discs, is capable of C20-hydroxylation of ecdysone to 20-hydroxyecdysone, as well as C26-hydroxylation, which results in 20,26-dihydroxyecdysone accumulating in the cells.[10]

Related studies on other epithelial-like cell lines have addressed the question of ecdysteroid synthetic capacity. For example, Ward et al. examined four lepidopteran and three orthopteran cell lines, and found ecdysteroid production most consistently in the UMBGE-4 cockroach chitin synthesizing cell line.[11] In addition, like the PID2 cells, both 20C- and 26C-hydroxylases were found in the cockroach cell line.[12] Similarly, Stiles et al. demonstrated that the BRL-AG-2 cell line derived from embryonic tissue of the cotton boll weevil is capable of both chitin synthesis and ecdysteroid production.[13] Interestingly, this cell line, like IAL-TND1, contained multicellular vesicles and aggregates. The relationship of epithelial-like capabilities and the ability to synthesize ecdysteroids also was found in dipteran as well as in orthopteran and lepidopteran cultures. A chitin-synthesizing cell line from *Chironomous tentans,* that also grows as multicellular vesicles, produced a material that cochromatographed with ecdysone.[14]

Taken together, these findings clearly show that cell lines that are capable of synthesizing chitin and grow as multicellular assemblages, or otherwise demonstrate an epithelial developmental determination, are capable of synthesizing ecdysteroids. These results support the overall hypothesis that there are alternate sites for ecdysteroid production *in vivo* and that these sites may "play a role in localized developmental events."[15]

B. ACTION OF ECDYSTEROIDS

1. Ecdysteroid Receptors and Response Elements

It is fair to state that without ecdysteroid responsive cell lines, progress toward unraveling the early events associated with hormonal action in insects would have been considerably delayed. Best-Belpomme, Cherbas, O'Connor, and colleagues were among the first to formally propose the use of cell lines as models for investigating the endocrine control of insect metamorphosis.[16,17,18] These authors all worked with the Kc cell line established from embryos of *Drosophila melanogaster.*[6] Recognition of the potential of these cell lines for studies of hormonal action came as the result of investigations by Courgeon, who was the first to observe effects of ecdysteroids on the morphology and rate of proliferation in the Kc cells.[6] These major responses of the Kc cell line to ecdysteroids led to the assumption that the mechanism of action of the hormone at the subcellular level in the Kc cells should be the same as that which operates *in vivo* even if the physiological meaning of the observations was not evident. Thus, while there may be some debate as to whether it is reasonable to derive insights on physiological processes from experiments performed on established cell lines, it is clearly advantageous to investigate the early effects of ecdysteroids in such systems.

A major contribution to our understanding of steroid hormone receptors in invertebrates was made as a result of the availability of high-specific-activity [^{3}H]-ponasterone A, which was used for characterizing ecdysteroid receptors in mass-isolated *D. melanogaster* imaginal discs[19] and in Kc cells.[20] The combination of a radioligand with greater affinity for the ecdysteroid receptor than 20-hydroxyecdysone, together with a plentiful supply of hormone-sensitive cells, provided the first demonstration of a single high-affinity saturable binding site for ecdysteroids. The relative K_D values are in the order of 10^{-7} M, ponasterone A, 10^{-7} to 10^{-8} M, 20-hydroxyecdysone, and 10^{-5} to 10^{-6} M, ecdysone.[21] Further improvements in this system were made by using [^{3}H] muristerone A as a radioligand in Kc cells.[22] The muristerone A receptor complex was more stable than other ecdysteroid receptor complexes and enabled the authors to characterize the receptor more fully and estimate its molecular weight at 120,000.

The view that there was only one binding site for ecdysteroids was revised as a consequence of research with another cell line from *D. melanogaster,* B II, which was used to demonstrate two high-affinity binding sites with kDas of 0.3×10^{-9} and 2×10^{-9}, based on the uptake of [^{3}H] ponasterone A by the intact cells. The presence of these high-affinity specific binding components was determined with Scatchard plots of binding data in cell-free extracts.[23] This work represents the first ecdysteroid receptor research with cell lines derived from a specific cell type, tumorous blood cells.[24] The presence of multiple binding sites for ponasterone A was later confirmed in intact adult *D. melanogaster* females.[25] Thus, *Drosophila* cell lines have been utilized to characterize ecdysteroid binding sites in concert with parallel studies *in vivo.*

Although there has been considerable emphasis on cell lines from *D. melanogaster,* other species also have been used for ecdysteroid receptor studies. For example, [^{3}H] ponasterone A was used as the radioligand in a *Chironomous tentans* cell line.[26,27] These authors demonstrated relative affinities for the binding component based on competition assays that supported the findings on *D. melanogaster* cells with regard to 20-hydroxyecdysone and ecdysone. Moreover, similar results were obtained by Wing, who used the *P. interpunctella* cell line, IAL-PID2, established by Lynn and Oberlander, to provide the first biochemical evidence for ecdysteroid receptors in lepidopteran cells.[28,29] [^{3}H] ponasterone A showed binding to nuclear extracts of the cells that was saturable and high affinity, while competitive binding with unlabeled hormone showed that 20-hydroxyecdysone and ecdysone displaced the radioligand, as reported for the dipteran systems. Thus, it is clear that a major portion of the progress that has been made in characterizing ecdysteroid receptors derives from research with established cell lines that retain their hormonal responsiveness.

Cell lines have been important tools not only in characterizing ecdysteroid receptors, but also in gaining insight into the early events that ensue in target cells after the hormone binds to its receptor. In this connection, the *D. melanogaster* cell lines have been critical biological material. Importantly, the Kc cell line displays little or no metabolism of 20-hydroxyecdysone,[20] so that the rapid degradation of this hormone *in vivo* is not a factor in the *in vitro* system. Moreover, morphological and biochemical responses of the Kc cell line to diverse ecdysteroids were similar to those reported for intact *D. melanogaster* tissues.[17]

Scientists believed that the Kc cells might provide information on the early effects of 20-hydroxyecdysone, and that looking for newly synthesized peptides would be the most useful approach. Ecdysteroid-induced peptides were characterized in the Kc cells just four hours after treatment of the cells with hormone. The ecdysteroid-induced peptides (EIPs) were designated EIP40, EIP29, and EIP28 to correspond to their molecular weights of 40,000, 29,000 and 28,000, respectively. It was possible to detect the EIPs only 30 minutes after the addition of ecdysteroid.[17,30] For an overview of ecdysteroid responsive genes in *D. melanogaster,* the reader is referred to Cherbas et al.,[31] a paper noteworthy for being the result of input from 27 coauthors.

Molecular analysis of gene expression in the EIP 28/29 region indicates that the corresponding mRNAs derive from four exons, and that differential use of alternative donor sites at the end of the second exon yields mRNAs that code for the two different proteins.[32] In a successful effort to map ecdysteroid response elements (EcREs), Cherbas et al.[33] utilized iodoponasterone A as the radioligand. They studied immobilized restriction fragments spanning a 9 kb genomic region that flanks the EIP 28/29 gene, and identified three specific fragments with autoradiography. One binding site mapped upstream of the transcription site while the other two mapped downstream. The two downstream sites were necessary for hormonal stimulation of the EIP 28/29 gene. Significantly, each of the three EcREs was able to function independently in conferring ecdysteroid responsiveness when located upstream of a gene that would otherwise not respond to hormone. Thus, over the course of two decades Courgeon's early observations of ecdysteroid responsiveness in the *Drosophila* Kc cell line led to a model system for characterizing ecdysteroid receptors and response elements.

During the same period in which Kc cells were investigated, other cell lines established from *D. melanogaster,* especially the S1, S2, and S3 lines, were important culture systems for molecular analysis.[34] These cell lines were used to identify an ecdysteroid response element in the regulatory region of a heat shock protein gene, *hsp* 27.[35] Just as with EIP 28/29 and EIP 40, the region required for ecdysteroid inducibility is located separately from the promoter region for the heat shock response. In an effort to unify these studies, Cherbas et al.[36] proposed a consensus sequence for ecdysone receptor binding, which is similar to response elements for receptors for some vertebrate hormones.[37]

Thus, a new understanding of ecdysteroid action at the molecular level has been obtained from intensive studies of *Drosophila* cell lines. At the same time, the *in vivo* significance of the EIP 28/29 gene product is not clear, although larval hemocytes and lymph gland showed ecdysteroid-induced activity of this gene *in vivo.*[38] The relationship of the small *hsp* genes to development *in vivo* is more apparent. Ecdysteroids induce the transcription of the four small heat shock genes in both the S3 cell line and in imaginal discs from pupariating larvae.[39,40] This may be the best indication that "the cultured cells represent a valid model system for studying...the developmental regulation of *hsp* gene activation."[41]

2. Stimulation of Enzymes and Glycoproteins

The same *Drosophila* cell lines that were so valuable in investigating the early effects of ecdysteroid action have also been of considerable use in characterizing hormonally induced enzymatic activities. Best-Belpomme et al.[16] reported that the activity of about 30 enzymes, including proteases, lipases, and kinases, did not change significantly when the Kc cells were exposed to 20-hydroxyecdysone, but that the activities of two enzymes, acetylcholinesterase and β-galactosidase, increased sharply.[42,43,44] In the case of the Kc line, acetylcholinesterase activity was virtually undetectable in the absence of hormone, but increased steadily after treatment with 20-hydroxyecdysone, reaching a peak on the third day. In another *Drosophila* cell line, derived from haploid embryos, untreated cells had a high level of acetylcholinesterase activity, but 20-hydroxyecdysone induced a sixfold increase in enzymatic activity even in this case.[16,45] Cherbas et al.[17] reported that a range of ecdysteroids induced acetylcholinesterase activity in Kc cells in roughly the same order of effectiveness as reported for an imaginal disc evagination assay, i.e., Ponasterone A > polypodine B > 20-hydroxyecdysone > inokosterone > ecdysone. These observations on the induction of enzymes by ecdysteroids in the Kc cells show that this cell line represents an excellent model system. The *in vivo* significance of the Kc cellular response to ecdysteroid appears to relate to hormonal effects on the lymph gland, a hematopoietic tissue, despite earlier notions that neuroblast cells may have contributed to this cell line.[18]

In terms of addressing questions of the consequences of ecdysteroid action beyond the early events, it would be helpful to measure biosynthetic activities that characterize development *in*

vivo. Recent efforts along these lines have attempted to link enzymatic activities and glycoprotein synthesis *in vitro* with chitin metabolism and morphogenesis *in vivo*. Sommer and Spindler[46] approached this problem by assaying chitin-degrading enzymatic activity in Kc cells. Earlier reports had measured chitinase activity from both lepidopteran and cockroach cell cultures.[47,48] The Kc cells were maintained in a serum-free medium, because chitinase activity is present in the vertebrate serum usually added to insect tissue culture media. This fact may obscure low levels of chitin synthesis in some insect cell lines. Sommer and Spindler found that β-*N*-acetyl-D-glucosaminidase and β-*N*-acetyl-D-hexosaminidase were produced spontaneously by the Kc cells and released into the medium. The products of their activity were *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine, both of which could inhibit the corresponding enzymatic activity. The authors suggest that this system would be advantageous for investigating ecdysteroid effects on chitin-degrading enzymes.

Related studies were conducted by Kramerov,[49] who reported that a *Drosophila* cell line secreted glycoproteins into the culture medium. This glycoprotein (110,000 Da) consisted of 4000-Da subunits, which contained a glycopeptide moiety bound to *N*-acetyl-D-glucosamine residues. Similar results were obtained for the Indian meal moth cell line, IAL-PID2. Porcheron et al.[50] approached this problem by purifying PID2-produced glycopeptides with gel-permeation HPLC. While glycosylated molecules were observed across the range of molecular weights evaluated, the untreated PID2 cells displayed major *N*-acetyl-D-glucosamine labeling in the 1500-dalton fraction. This peak shifted to the 5000-Da region after treatment of the cell line with 20-hydroxyecdysone, a finding confirmed with dual labeling with radiolabeled leucine and *N*-acetyl-D-glucosamine. Moreover, a glycopeptide of similar molecular weight was observed in ecdysteroid-treated imaginal discs. Although the function of this glycopeptide is not known, we suspect it may play a role in cuticle formation, if only because the potent chitin synthesis inhibitor, teflubenzuron, prevented its appearance. In any event, the presence of a 5000-Da glycopeptide following ecdysteroid treatment in intact imaginal discs as well as in the cell line suggests that this molecule represents expression of differentiated function.

In addition to a role in cuticle formation, extracellular glycoproteins are reported to be important in imaginal disc morphogenesis (evagination), which depends upon cell rearrangement.[51,52] As mentioned earlier in connection with research on the small heat shock genes of the Schneider *Drosophila* cell lines, these cell lines have synthetic activities that typify imaginal discs. In this context Rickoll and Galewsky[53] demonstrated that the S3 cell line secreted glycoproteins into the culture medium, and that 20-hydroxyecdysone increased the labeling of these proteins. Moreover, three of the ecdysteroid-dependent secreted glycoproteins (116,000, 110,000, 80,000 daltons) were immunoprecipitated with an antiserum raised against cell membranes from *Drosophila* imaginal discs. Ecdysteroid-induced aggregation of the S3 cells was correlated with the appearance of some of these glycoproteins. The possibility of involvement of these glycoproteins in cuticle formation was not assessed by the authors, but there is a strong supposition that these hormone-directed syntheses during S3 cell reaggregation may reflect the cell-cell interactions that occur during imaginal disc morphogenesis.

3. Chitin Synthesis

The biochemistry of cuticle formation in insects has attracted the attention of surprisingly few scientists, perhaps because the complex structure of the cuticle obscures the molecular interactions that give rise to the insect exoskeleton. Morphological analysis points to the apical microvilli, "epidermal feet," as the site of synthesis of chitin, the major nonproteinaceous constituents of cuticle.[54] Interest in the hormonal regulation of chitin synthesis, as well as the mode of action of benzoylphenyl ureas that inhibit chitin synthesis, makes the availability of chitin-synthesizing cell lines a significant priority.

There is evidence that a chitin-like material is synthesized spontaneously by a variety of cell lines. Marks and Ward[55] checked for incorporation of radio-labeled *N*-acetyl-D-glucosamine

into embryo-derived cell lines of the cockroach *Blatella germanica* (UMBGE-4), the moths *Manduca sexta* (MRRL-CH 34), and *Plodia interpunctella* (UMN-PIE), as well as cell lines derived from imaginal discs of *P. interpunctella* (IAL-PID2) and *Trichoplusia ni* (IAL-TND1). In all cases at least some of the samples produced a chitin-like material. Evidence that the UMBGE-4 product that incorporated *N*-acetyl-D-glucosamine was indeed chitin was based on its ability to withstand digestion for one hour at 100°C in 1 *N* NaOH, and that it was degraded by chitinase to chitobiose and *N*-acetyl-D-glucosamine. Material from the other cell lines meet all of these criteria for chitin.[56,57] In addition to orthopteran and lepidopteran cell lines, two dipteran cell lines, the *Drosophila* Kc line and a *C. tentans* line, showed evidence of chitin synthesis.[58]

Interestingly, cell lines that display some capability for synthesizing chitin-like materials often grow as multicellular vesicles, e.g., the UMBGE-4 cell line, the *C. tentans* epithelial cell line,[27,58] and the *T. ni* imaginal disc cell line.[8] However, this is not an absolute requirement for synthesis of chitin-like materials, since the *Drosophila* Kc cells and the IA1-PID2 cells grow as a monolayer.

The influence of ecdysteroids on chitin synthesis and related activities has been tested in several cell lines. The addition of 20-hydroxyecdysone increases the level of chitin synthesis in the UMBGE cell line,[56] although it depresses chitin synthesis in the *C. tentans* cell line.[58,59] In a related study, the stimulation by ecdysteroids of the uptake of the chitin precursor, *N*-acetyl-D-glucosamine, was investigated in the imaginal disc-derived cell line, IAL-PID2.[60] This reliable response to ecdysteroids was not inhibited by benzoylphenyl urea compounds that inhibit chitin synthesis *in vivo*,[60] although chitin synthesis in the UMBGE-4 cell line was blocked by diflubenzuron.[56] These results suggest that uptake of chitin precursors is not a site of action for benzoylphenyl ureas. Although the mode of action of such inhibitors remains obscure, chitin-producing cell lines should be important experimental models in such research.

III. MORPHOGENESIS

A. REGULATION OF MORPHOLOGY

1. Hemolymph and Tissue Factors

The most obvious morphological characteristic of the *Trichoplusia ni* cell line, IAL-TND1, was that it grew as fluid-filled multicellular spheres.[29] This was a cell line that apparently was derived from the epithelial cells of wing imaginal discs. One of the tests run on these cells compared the 2-D gel protein patterns of the cell line with imaginal discs, fat body, and hemocytes from the insect. The protein pattern for the cell line was virtually identical to intact imaginal discs and distinct from the pattern for other cell types that could have contaminated the primary cultures. This result, which was a control for confirming the source of the cell line, may be among the best evidence for expression of differentiated function by an insect cell line. Consider the definition of differentiation by Jacob and Monod, as quoted by Gilbert,[62] "Two cells are differentiated with respect from one another if, while they harbor the same genome, the pattern of proteins they synthesize is different." The maintenance of differentiative capacity was demonstrated when these spheres were transplanted back into larvae and underwent metamorphosis with the host, where they were recovered as pupal wings with tanned cuticle.[78]

Although we do not know the *in vivo* significance of the vesicle form that both the IAL-SPD1 and IAL-TND1 cultures took, we were confronted with a spontaneous change of the vesicles to amorphous aggregates. We found that larval hemolymph caused a reversal of this morphology back to the multicellular vesicle form. A factor was partially purified from larval hemolymph of the cabbage looper, *Trichoplusia ni*, that caused cell aggregates of an imaginal disc cell line, IAL-TND1, to form multicellular vesicles.[63,64] The vesicle-promoting factor (VPF) was characterized as a nonglycosylated polypeptide with a 16.9 kDa molecular weight.

Interestingly, we found that the action of VPF can be thwarted by coincubation of the cells with ecdysteroids. While the role of VPF *in vivo* remains unclear at this time, it may well represent one of a number of growth and morphogenetic factors that will be uncovered by use of tissue-culture systems.

2. Hormonal Effects

Cellular elongation is a typical response to ecdysteroids in both dipteran and lepidopteran cell lines.[6,65] For example, the *Manduca* cell line, MRRL-CH1, derived from embryos, responds to ecdysteroids by extensive elongation of the cells, and it does so in a dose-dependent manner.[66] Thus, we have three processes that may relate to expression of differentiated function. First, the cells do respond to ecdysteroids; second, there is a marked change in morphology of the cells that typifies this response, and third, as the work on subclones shows, there is maintenance of cellular diversity; i.e., some cells and their progeny respond well to 20-hydroxyecdysone, and some respond very poorly. Moreover, this response requires the synthesis of proteins for the ecdysteroid to have any effect.[67] Similar effects of ecdysteroids on cellular elongation in lepidopteran cell lines were obtained for TN-368 *(Trichoplusia ni)* and HPB-SL-26 *(Spodoptera littoralis).*[68] Thus, we have several cases in which the overt effect of the hormone on the cells is cellular elongation, and in which the other criteria for expression of differentiated function may come into play. Still, because we do not know the *in vivo* cell types represented here, nor do we know the developmental significance of this cellular elongation, there may be doubt as to the validity of this morphological response as an example of differentiated function.

Research with an imaginal disc-derived cell line derived from *Plodia interpunctella,* IAL-PID2, provided a quite different morphology, basically consisting of fibroblast-like cells. The wing discs in the primary cultures were cut repeatedly, resulting in both vesicular blebs and a monolayer outgrowth, which became the source of this cell line.[29] The PID2 cell line is a vigorous, rather typical cell line, without any striking multicellular formations. However, these cells have demonstrated some interesting properties, among these the ability to respond to ecdysteroids with decreased proliferation[69] and increased uptake of *N*-acetyl-glucosamine from the medium.[60] This ecdysteroid-dependent response is characteristic of an early effect of 20-hydroxyecdysone on intact imaginal wing discs at the start of metamorphosis. Thus, we have a clear demonstration of the expression of a developmental capacity that represents an early step in preparation for cuticle synthesis.

A detailed morphological study revealed significant changes in PID2 cells in response to ecdysteroids. Under hormone-free conditions the cells grow as a monolayer of round or spindle-shaped cells. Moreover, localization of wheatgerm agglutinin-gold particles showed that there were glycosylation sites only along the cellular extensions of untreated cells.[70] After treatment with 20-hydroxyecdysone, the cells formed epithelial-like aggregates that were interconnected and linked by desmosomes. In hormone-treated cells, WGA-binding sites were present not only along the cellular extensions, but also along the entire plasma membrane. This pronounced shift in the distribution of glycosylation sites perhaps suggests preparation for ecdysteroid-induced differentiation, including cuticle formation. Thus, the PID2 cells respond to ecdysteroids in a manner that is meaningful in terms of the development *in vivo* of the imaginal discs from which they were derived.

In some cell lines it may be obvious from the morphological response to hormones that a developmental phenomenon with *in vivo* significance has been obtained, despite not having a clear definition of their cellular origin. For example, a cell line derived from the hymenopteran egg parasitoid, *Trichogramma exiguum* (IPLB-Tex2), produces highly contractile cells in response to 20-hydroxyecdysone.[71] This observation suggests that the cell line may be composed of myoblast cells. Similarly, the spontaneous formation of contractile cells from a cell line was noted in NISES-AnPe-426 *(Antheraea pernyi)* after several months in culture.[72]

B. CELL LINES AS SOURCES OF DEVELOPMENTAL FACTORS

We will turn now to cell lines that display *in vivo* properties through the production of specific products utilized by other cells. An example of this situation comes from the cell line that was established from fat body of the gypsy moth.[73] This cell line is quite different in morphology from the imaginal disc-derived cell lines that we have been discussing. As one of a relatively small number of cell lines derived from a specific cell type, the LdFB cells may have maintained differentiated function. We speculated that the cell line might substitute for fat body in promoting the development of parasitoids grown in tissue culture. In this connection, we tested the possibility that the LdFB cells could affect the development of *Microplitis croceipes,* a beneficial insect. Embryogenesis and hatching of this parasite is clearly stimulated by fat body-conditioned culture medium.[74] Also, we demonstrated that the LdFB cells can substitute for larval host fat body in stimulating egg development of the parasite. The active substance(s) that induced germ-band development was dialyzable (<10 K), while the hatch-stimulating factor was nondialyzable (>10K). Both active materials were resistant to trypsin and to heat. Eggs held in media conditioned with the cell line responded in a dose-dependent manner. Manifestly, this is a dramatic example of a cell line retaining differentiated function by producing a specific product that has a developmental effect on another species.

In other culture systems, a combination of vertebrate growth factors and conditioned medium from insect cell lines leads to physiological functioning of cultured tissues, as in the case of the venom gland of the ant *Pseudomyrmex triplarinus.* This gland was cultured in medium formulated with epidermal growth factor, fibroblast growth factor, insulin, cAMP, cGMP, and isoproterenol,[75] but the medium had to be conditioned with established lepidopteran cell lines for long-term cell survival. The metabolic production of the venom was maintained for 12 months. Similarly, conditioned medium from a *Manduca sexta* cell line could substitute for various tissues in stimulating neuronal outgrowth and glial cell survival in cultures from the antennal lobe of *M. sexta* brains.[76]

IV. CONCLUSIONS AND PROSPECTS

We have seen examples of maintenance of differentiated function in cell lines derived from embryos, imaginal discs, and fat body. In general, the extra effort to obtain cell lines from specific tissue types rewards the investigator with cells that are more robust in their response to hormones, developmental capacity, and ability to produce specific cell products.[77] We have only seen a glimpse of what can be accomplished with cell lines that retain a rigorous fidelity to their *in vivo* origin.

We would like to close by sharing with you some of the reasons we believe there is now increasing success in obtaining cell lines from specific insect tissues that retain some measure of differentiated function. The most important ingredient is patience, not only staying with a problem but learning to ignore it artfully, employing what Tom Grace called "organized neglect" (quoted by Maramorosch[2]). Thus, one may wait a year or more in these cases for a spontaneous immortalizing event. Second, the medium must, at the least, keep the cells alive and foster proliferation — a problem solved for many systems with Grace's medium that was based in turn on Wyatt's analysis of insect hemolymph. Third, the investigator should think of the *in vitro* culture system as an organism — with a plastic or glass exoskeleton, an open circulatory system, a requirement for oxygen, and a need to be protected from desiccation, microbes, and predators (including humans); put another way, the tissue culturist must learn to think like a petri dish! Fourth, the investigator needs to understand as much as possible about the cellular ecology *in vivo,* so that conditions can be created in which the cells will respond to appropriate signals with responses that are meaningful in terms of their *in vivo* origin.

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